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(54) POLYMER-PEPTIDE CONJUGATES

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Description

Field of the Invention

[0001] The present invention relates to polymer-peptide conjugates and formulations, and to methods of making and using same.

Description of the Related Art

[0002] The use of polypeptides and proteins for the systemic treatment of certain diseases is now well accepted in medical practice. The role that the peptides play in replacement therapy is so important that many research activities are being directed towards the synthesis of large quantities by recombinant DNA technology. Many of these polypeptides are endogenous molecules which are very potent and specific in eliciting their biological actions.

[0003] A major factor limiting the usefulness of these substances for their intended application is that they are easily metabolized by plasma proteases when given parenterally. The oral route of administration of these substances is even more problematic because in addition to proteolysis in the stomach, the high acidity of the stomach destroys them before they reach their intended target tissue. Polypeptides and protein fragments, produced by the action of gastric and pancreatic enzymes, are cleaved by exo and endopeptidases in the intestinal brush border membrane to yield di- and tripeptides, and even if proteolysis by pancreatic enzymes is avoided, polypeptides are subject to degradation by brush border peptidases. Any of the given peptides that survive passage through the stomach are further subjected to metabolism in the intestinal mucosa where a penetration barrier prevents entry into the cells.

[0004] In spite of these obstacles, there is substantial evidence in the literature to suggest that nutritional and pharmaceutical proteins are absorbed through the intestinal mucosa. On the other hand, nutritional and drug (poly)peptides are absorbed by specific peptide transporters in the intestinal mucosa cells. These findings indicate that properly formulated (poly)peptides and proteins may be administered by the oral route, with retention of sufficient biological activity for their intended use. If, however, it were possible to modify these peptides so that their physiological activities were maintained totally, or at least to a significant degree, and at the same time stabilize them against proteolytic enzymes and enhance their penetration capability through the intestinal mucosa, then it would be possible to utilize them properly for their intended purpose. The product so obtained would offer advantages in that more efficient absorption would result, with the concomitant ability to use lower doses to elicit the optimum therapeutic effect.

[0005] The problems associated with oral or parenteral administration of proteins are well known in the pharmaceutical industry, and various strategies are being used in attempts to solve them. These strategies include incorporation of penetration enhancers, such as the salicylates, lipid-bile salt-mixed micelles, glycerides, and acylcamitines, but these frequently are found to cause serious local toxicity problems, such as local irritation and toxicity, complete abrasion of the epithelial layer and inflammation of tissue. These problems arise because enhancers are usually co-administered with the peptide product and leakages from the dosage form often occur. Other strategies to improve oral delivery include mixing the peptides with protease inhibitors, such as aprotinin, soybean trypsin inhibitor, and amastatin, in an attempt to limit degradation of the administered therapeutic agent. Unfortunately these protease inhibitors are not selective, and endogenous proteins are also inhibited. This effect is undesirable.

[0006] Enhanced penetration of peptides across mucosal membranes has also been pursued by modifying the physicochemical properties of candidate drugs. Results indicate that simply raising lipophilicity is not sufficient to increase paracellular transport. Indeed it has been suggested that cleaving the peptide-water hydrogen bonds is the main energy barrier to overcome in obtaining peptide diffusion across membranes (Conradi, R. A., Hilgers, A.R., Ho, N.F.H., and Burton, P.S., "The influence of peptide structure on transport across Caco-2 cells", *Pharm. Res.,* 8, 1453-1460, (1991)). Protein stabilization has been described by several authors. Abuchowski and Davis ("Soluble polymers-Enzyme adducts", In: *Enzymes* as *Drugs,* Eds. Holcenberg and Roberts, J. Wiley and Sons, New York, NY, (1981)) disclosed various methods of derivatization of enzymes to provide water soluble, non-immunogenic, *in vivo* stabilized products.

[0007] A great deal of work dealing with protein stabilization has been published. Abuchowski and Davis disclose various ways of conjugating enzymes with polymeric materials (Ibid.). More specifically, these polymers are dextrans, polyvinyl pyrrolidones, glycopeptides, polyethylene glycol and polyamino acids. The resulting conjugated polypeptides are reported to retain their biological activities and solubility in water for parenteral applications. The same authors, in U.S. Patent No. 4,179,337, disclose that polyethylene glycol rendered proteins soluble and non-immunogenic when coupled to such proteins. These polymeric materials, however, did not contain fragments suited for intestinal mucosa binding, nor did they contain any moieties that would facilitate or enhance membrane penetration. While these conjugates were water-soluble, they were not intended for oral administration.

[0008] Meisner et al., U.S. Patent No. 4,585,754, teaches that proteins may be stabilized by conjugating them with chondroitin sulfates. Products of this combination are usually polyanionic, very hydrophilic, and lack cell penetration capability. They are usually not intended for oral administration.

[0009] Milt et al., U. S. Patent 4,003,792, teaches that certain acidic polysaccharides, such as pectin, algesic acid, hyaluronic acid and carrageenan, can be coupled to proteins to produce both soluble and insoluble products. Such polysaccharides are polyanionic, derived from food plants. They lack cell penetration capability and are usually not intended for oral administration.

[0010] In Pharmacological Research Communication 14, 11-120 (1982), Boccu et al. disclosed that polyethylene glycol could be linked to a protein such as superoxide dismutase ("SOD"). The resulting conjugated product showed increased stability against denaturation and enzymatic digestion. The polymers did not contain moieties that are necessary for membrane interaction and thus suffer from the same problems as noted above in that they are not suitable for oral administration.

[0011] Other techniques of stabilizing peptide and protein drugs in which proteinaceous drug substances are conjugated with relatively low molecular weight compounds such as aminolethicin, fatty acids, vitamin B_{12}, and glycosides, are revealed in the following articles: R. Igarishi et al., "Proceed. Intern. Symp. Control. Rel. Bioact. Materials, 17, 366, (1990);T. Taniguchi et al. Ibid 19, 104, (1992); G. J. Russel-Jones, Ibid, 19, 102, (1992); M. Baudys et al., Ibid, 19, 210, (1992). The modifying compounds are not polymers and accordingly do not contain moieties necessary to impart both the solubility and membrane affinity necessary for bioavailability following oral as well as parenteral administration. Many of these preparations lack oral bioavailability.

[0012] Another approach which has been taken to lengthen the *in vivo* duration of action of proteinaceous substances is the technique of encapsulation. M. Saffan et al., in Science, 223, 1081, (1986) teaches the encapsulation of proteinaceous drugs in an azopolymer film for oral administration. The film is reported to survive digestion in the stomach but is degraded by microflora in the large intestine, where the encapsulated protein is released. The technique utilizes a physical mixture and does not facilitate the absorption of released protein across the membrane.

[0013] Ecanow, U.S. Patent No. 4,963,367, teaches that physiologically active compounds, including proteins, can be encapsulated by a coacervative-derived film and the finished product can be suitable for transmucosal administration. Other formulations of the same invention may be administered by inhalation, oral, parenteral and transdermal routes. These approaches do not provide intact stability against acidity and proteolytic enzymes of the gastrointestinal tract, the property as desired for oral delivery.

[0014] Another approach taken to stabilize protein drugs for oral as well as parenteral administration involves entrapment of the therapeutic agent in liposomes. A review of this technique is found in Y. W. Chien, "New Drug Delivery Systems", Marcel Dekker, New York, NY, 1992. Uposome-protein complexes are physical mixtures; their administration gives erratic and unpredictable results. Undesirable accumulation of the protein component in certain organs has been reported, in the use of such liposome-protein complexes. In addition to these factors, there are additional drawbacks associated with the use of liposomes, such as cost, difficult manufacturing processes requiring complex lypophilization cycles, and solvent incompatibilities. Moreover, altered biodistribution and antigenicity issues have been raised as limiting factors in the development of clinically useful liposomal formulations.

[0015] The use of "proteinoids" has been described recently (Santiago, N., Milstein, S. J., Rivera, T., Garcia, E., Chang., T.C., Baughman, R.A., and Bucher, D., "Oral Immunization of Rats with Influenza virus M Protein (M1) Microspheres", *Abstract #A 221, Proc. Int. Symp. Control. Rel. Bioac. Mater.*, **19**, 116 (1992)). Oral delivery of several classes of therapeutics has been reported using this system, which encapsulates the drug of interest in a polymeric sheath composed of highly branched amino acids. As is the case with liposomes, the drugs are not chemically bound to the proteinoid sphere, and leakage of drug out of the dosage form components is possible.

[0016] A peptide which has been the focus of much synthesis work, and efforts to improve its administration and bioassimilation, is insulin.

[0017] The use of insulin as a treatment for diabetes dates back to 1922, when Banting et al. ("Pancreatic Extracts in the Treatment of Diabetes Mellitus," Can. Med. Assoc. J., 12, 141-146 (1922)) showed that the active extract from the pancreas had therapeutic effects in diabetic dogs. Treatment of a diabetic patient in that same year with pancreatic extracts resulted in a dramatic, life-saving clinical improvement. A course of daily injections of insulin is required for extended recovery.

[0018] The insulin molecule consists of two chains of amino acids linked by disulfide bonds; the molecular weight of insulin is around 6,000. The β-cells of the pancreatic islets secrete a single chain precursor of insulin, known as proinsulin. Proteolysis of proinsulin results in removal of four basic amino acids (numbers 31, 32, 64 and 65 in the proinsulin chain: Arg, Arg, Lys, Arg respectively) and the connecting ("C") peptide. In the resulting two-chain insulin molecule, the A chain has glycine at the amino terminus, and the B chain has phenylalanine at the amino terminus.

[0019] Insulin may exist as a monomer, dimer or a hexamer formed from three of the dimers. The hexamer is coordinated with two Zn^{2+} atoms. Biological activity resides in the monomer. Although until recently bovine and porcine insulin were used almost exclusively to treat diabetes in humans, numerous variations in insulin between species are known. Porcine insulin is most similar to human insulin, from which it differs only in having an alanine rather than threonine residue at the B-chain C-terminus. Despite these differences most mammalian insulin has comparable specific activity. Until recently animal extracts provided all insulin used for treatment of the disease. The advent of recom-

binant technology allows commercial scale manufacture of human insulin (e.g., Humulin™ insulin, commercially available from Eli Lilly and Company, Indianapolis, IN).

[0020] Although insulin has now been used for more than 70 years as a treatment for diabetes, few studies of its formulation stability appeared until two recent publications (Brange, J., Langkjaer, L., Havelund, S., and Vølund, A., "Chemical stability of insulin. I. Degradation during storage of pharmaceutical preparations," *Pharm. Res.,* 9, 715-726, (1992); and Brange, J. Havelund, S., and Hougaard, P., "Chemical stability of insulin. 2. Formulation of higher molecular weight transformation products during storage of pharmaceutical preparations," *Pharm. Res.,* 9, 727-734, (1992)). In these publications, the authors exhaustively describe chemical stability of several insulin preparations under varied temperature and pH conditions. Earlier reports focused almost entirely on biological potency as a measure of insulin formulation stability. However the advent of several new and powerful analytical techniques - disc electrophoresis, size exclusion chromatography, and HPLC - allows a detailed examination of insulin's chemical stability profile. Early chemical studies on insulin stability were difficult because the recrystallized insulin under examination was found to be no more than 80-90% pure. More recently monocomponent, high-purity insulin has become available. This monocomponent insulin contains impurities at levels undetectable by current analysis techniques.

[0021] Formulated insulin is prone to numerous types of degradation. Nonenzymatic deamidiation occurs when a side-chain amide group from a glutaminyl or asparaginyl residue is hydrolyzed to a free carboxylic acid. There are six possible sites for such deamidiation in insulin: Gln^{A5}, Gln^{A15}, Asn^{A18}, Asn^{A21}, Asn^{B3}, and Gln^{B4}. Published reports suggest that the three Asn residues are most susceptible to such reactions.

[0022] Brange et al. (ibid) reported that in acidic conditions insulin is rapidly degraded by extensive deamidation at Asn^{A21}. In contrast, in neutral formulations deamidation takes place at Asn^{B3} at a much slower rate, independent of insulin concentration and species of origin of the insulin. However, temperature and formulation type play an important role in determining the rate of hydrolysis at B3. For example, hydrolysis at B3 is minimal if the insulin is crystalline as opposed to amorphous. Apparently the reduced flexibility (tertiary structure) in the crystalline form slows the reaction rate. Stabilizing the tertiary structure by incorporating phenol into neutral formulations results in reduced rates of deamidation.

[0023] In addition to hydrolytic degradation products in insulin formulations, high molecular weight transformation products are also formed. Brange et al. showed by size exclusion chromatography that the main products formed on storage of insulin formulations between 4 and 45°C are covalent insulin dimers. In formulations containing protamine, covalent insulin protamine products are also formed. The rate of formulation of insulin-dimer and insulin-protamine products is affected significantly by temperature. For human or porcine insulin, (regular N1 preparation) time to formation of 1% high molecular weight products is decreased from 154 months to 1.7 months at 37°C compared to 4°C. For zinc suspension preparations of porcine insulin, the same transformation would require 357 months at 4°C but only 0.6 months at 37°C.

[0024] These types of degradation in insulin may be of great significance to diabetic subjects. Although the formation of high molecular weight products is generally slower than the formation of hydrolytic (chemical) degradation products described earlier, the implications may be more serious. There is significant evidence that the incidence of immunological responses to insulin may result from the presence of covalent aggregates of insulin (Robbins, D.C. Cooper, S. M. Fineberg, S.E., and Mead, P.M., "Antibodies to covalent aggregates of insulin in blood of insulin-using diabetic patients", *Diabetes,* 36, 838-841, (1987); Maislos, M., Mead, P.M., Gaynor, D.H., and Robbins, D.C., "The source of the circulating aggregate of insulin in type I diabetic patients is therapeutic insulin", *J. Clin. Invest.*, 77, 717-723. (1986); and Ratner R. E., Phillips, T. M., and Steiner, M., "Persistent cutaneous insulin allergy resulting from high molecular weight insulin aggregates", *Diabetes*, 39, 728-733, (1990)). As many as 30% of diabetic subjects receiving insulin show specific antibodies to covalent insulin dimers. At a level as low as 2% it was reported that the presence of covalent insulin dimers generated a highly significant response in lymphocyte stimulation in allergic patients. Responses were not significant when dimer content was in the range 0.3-0.6%. As a result it is recommended that the level of covalent insulin dimers present in formulation be kept below 1% to avoid clinical manifestations.

[0025] Several insulin formulations are commercially available; although stability has been improved to the extent that it is no longer necessary to refrigerate all formulations, there remains a need for insulin formulations with enhanced stability. A modified insulin which is not prone to formation of high molecular weight products would be a substantial advance in the pharmaceutical and medical arts, and modifications providing this stability (and in addition providing the possibility of oral availability of insulin) would make a significant contribution to the management of diabetes.

[0026] In addition to the *in vivo* usage of polypeptides and proteins as therapeutic agents, polypeptides and proteins also find substantial and increasing use in diagnostic reagent applications. In many such applications, polypeptides and proteins are utilized in solution environments wherein they are susceptible to thermal and enzymic degradation of (poly)peptides and proteins such a enzymes, peptide and protein hormones, antibodies, enzyme-protein conjugates used for immunoassay, antibody-hapten conjugates, viral proteins such as those used in a large number of assay methodologies for the diagnosis or screening of diseases such as AIDS, hepatitis, and rubella, peptide and protein growth factors used for example in tissue culture, enzymes used in clinical chemistry, and insoluble enzymes such as

those used in the food industry. As a further specific example, alkalin phosphatase is widely utilized as a reagent in kits used for the colorimetric detection of antibody or antigen in biological fluids. Although such enzyme is commercially available in various forms, including free enzyme and antibody conjugates, its storage stability and solution often is limited. As a result, alkalin phosphatase conjugates are frequently freeze-dried, and additives such as bovine serum albumin and Tween 20 are used to extend the stability of the enzyme preparations. Such approaches, while advantageous in some instances to enhance the resistance to degradation of the polypeptide and protein agents, have various shortcomings which limit their general applicability.

SUMMARY OF THE INVENTION

[0027] The present invention relates generally to polymer-peptide conjugates and formulations, and to methods of making and using same.

[0028] More particularly, the present invention relates in one broad compositional aspect to covalently conjugated peptide complexes wherein the peptide is covalently bonded to one or more molecules of a polymer incorporating as an integral part thereof a hydrophilic moiety, e.g., a linear polyalkylene glycol, and wherein said polymer incorporates a lipophilic moiety as an integral part thereof.

[0029] In one particular aspect, the present invention relates to a physiologically active peptide composition comprising a physiologically active peptide covalently coupled with a polymer comprising (i) a linear polyalkylene glycol moiety and (ii) a lipophilic moiety, wherein the peptide, linear polyalkylene glycol moiety, and the lipophilic moiety are conformationally arranged in relation to one another such that the physiologically active peptide in the physiologically active peptide composition has an enhanced *in vivo* resistance to enzymatic degradation, relative to the physiologically active peptide alone (i.e., in an unconjugated form devoid of the polymer coupled thereto).

[0030] The term "peptide" as used herein is intended to be broadly construed as inclusive of polypeptides *per se* having molecular weights of up to about 10,000, as well as proteins having molecular weights of greater than about 10,000, wherein the molecular weights are number average molecular weights. As used herein, the term "covalently coupled" means that the specified moieties are either directly covalently bonded to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. The term "conjugatively coupled" means that the specified moieties are either covalently coupled to one another or they are non-covalently coupled to one another, e.g., by hydrogen bonding, ionic bonding, Van der Waals forces, etc.

[0031] The invention thus comprehends various compositions for therapeutic (*in vivo*) application, wherein the peptide component of the conjugated peptide complex is a physiologically active, or bioactive, peptide. In such peptide-containing compositions, the conjugation of the peptide component to the polymer comprising hydrophilic and lipophilic moieties may be direct covalent bonding or indirect (through appropriate spacer groups) bonding, and the hydrophilic and lipophilic moieties may also be structurally arranged In the polymeric conjugating structure in any suitable manner involving direct or indirect covalent bonding, relative to one another. Thus, a wide variety of peptide species may be accommodated in the broad practice of the present invention, as necessary or desirable in a given end use therapeutic application.

[0032] In another aspect, covalently coupled peptide compositions such as those described above may utilize peptide components intended for diagnostic or *in vitro* applications, wherein the peptide is for example a diagnostic reagent, a complement of a diagnostic conjugate for immunoassay or other diagnostic or non-*in vivo* applications. In such non-therapeutic applications, the peptide complexes of the invention are highly usefully employed as stabilized compositions which may for example be formulated in compatible solvents or other solution-based formulations to provide stable compositional forms which are of enhanced resistance to degradation.

[0033] In the foregoing therapeutic and non-therapeutic (e.g., diagnostic) applications, the present invention relates in a broad compositional aspect to covalently conjugated peptide complexes wherein the peptide is covalently bonded to one or more molecules of a polymer incorporating as an integral part of said polymer a hydrophilic moiety, e.g., a polyalkylene glycol moiety, and a lipophilic moiety, e.g., a fatty acid moiety. In one preferred aspect, the peptide may be covalently conjugated by covalent bonding with one or more molecules of a linear polyalkylene glycol polymer incorporated in which as an integral part thereo is a lipophilic moiety, e.g., a fatty acid moiety.

[0034] In another particular broad aspect, the present invention relates to non-covalently conjugated peptide complexes wherein the peptide is non-covalently associated with one or more molecules of a polymer incorporating as an integral part thereof a hydrophilic moiety, e.g., a polyalkylene glycol moiety, and a lipophilic moiety, e.g., a fatty acid moiety. The polymer may be variously structured and arranged analogous to description of the polymer in the covalently conjugated peptide complexes described above, but wherein the peptide is not bonded to the polymer molecule(s) in a covalent manner, but is nonetheless associated with the polymer, as for example by associative bonding, such as hydrogen bonding, ionic bonding or complexation, Van der Waals bonding, micellular encapsulation or association (of the specific peptide), etc.

[0035] Such non-covalent associations of a peptide component and polymeric moiety/(ies) may for example utilize a peptide component for therapeutic (e.g., *in vivo*) applications, as well as non-therapeutic peptide components, e.g., for diagnostic or other *(in vitro*) use.

[0036] In such associatively conjugated peptide compositions, the polymer component may be suitably constructed, modified, or appropriately functionalized to impart the ability for associative conjugation in a selectively manner (for example, to impart hydrogen bonding capability to the polymer viz-a-vis the peptide), within the skill of the art.

[0037] Other aspects, features, and modifications of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038]

Figure 1 is a graph of serum glucose, in mg/dL, as a function of time, in minutes, for administration of insulin per se and in complexed forms.

Figure 2 is a graph of serum glucose, in mg/dL, as a function of time, in hours, for administration of insulin in various forms.

Figure 3 is a graph of chymotrypsin digestion of insulin and OT insulin as a function of time.

Figure 4 is a graph of serum calcium in rats as a function of time when the rats were administered orally polymer calcitonin conjugates.

Figure 5 is a bar graph showing serum calcium levels of rats as a function of time, when the rats were orally administered polymer calcitonin OT1-ct or OT2-ct polymer calcitonin conjugates.

Figure 6 shows a bar graph of the effects of polymer-insulin in diabetic rat model.

Figure 7 is a graph which shows the effect of orally administered 001-insulin in cynomolgous monkeys as a percent change in blood glucose over time.

Figure 8 is a bar graph which shows the effect of orally administered 001-insulin in cynomolgous monkeys as an effect of the dose over time.

DETAILED DESCRIPTION OF THE INVENTION, AND PREFERRED EMBODIMENTS THEREOF

[0039] Modification of peptides with non-toxic, non-immunogenic polymers may offer certain advantages. If modifications are made in such a way that the products (polymer-peptide conjugates) retain all or most of their biological activities the following properties may result: epithelial penetration capability may be enhanced; the modified peptide may be protected from proteolytic digestion and subsequent abolition of activity; affinity for endogenous transport systems may be improved; chemical stability against stomach acidity may be imparted; the balance between lipophilicity and hydrophobicity of the polymers may be optimized. Proteinaceous substances endowed with the improved properties described above may be effective as replacement therapy following either oral or parenteral administration. Other routes of administration, such as nasal and transdermal, may also be possible using the modified peptide.

[0040] In non-therapeutic applications, conjugation-stabilization of diagnostic and/or reagent species of peptides, including precursors and intermediates of end-use peptide or other products, provides corresponding advantages, when the peptide component is covalently bonded to a polymer in the manner of the present invention. The resultingly covalently conjugated peptide is resistant to environmental degradative factors, including solvent- or solution-mediated degradation processes. As a result of such enhanced resistance to degradation, the shelf life of the active peptide ingredient is able to be significantly increased, with concomitant reliability of the peptide-containing composition in the specific end use for which same is employed.

[0041] The covalent conjugation of peptides with polymers in the manner of the present invention effectively minimizes hydrolytic degradation, and achieves *in vitro* and *in vivo* stabilization.

[0042] Analogous benefits are realized when therapeutic, diagnostic, or reagent species peptides are non-covalently, associatively conjugated with polymer molecule(s) in the manner of the present invention.

[0043] Utilizing insulin covalently bonded to the polymer component as an illustrative embodiment of the invention, the nature of the conjugation, involving cleavable covalent chemical bonds, allows for control in terms of the time course

over which the polymer may be cleaved from the peptide (insulin). This cleavage may occur by enzymatic or chemical mechanisms. The conjugated polymer-peptide complex will be intrinsically active. Full activity will be realized following enzymatic cleavage of the polymer from the peptide. Further, the chemical modification will allow penetration of the attached peptide, e.g., insulin, through cell membranes. In a preferred aspect of the present invention, membrane penetration-enhancing properties of lipophilic fatty acid residues are incorporated into the body of the conjugating polymer. In this respect, again utilizing insulin as the peptide of interest, fatty acid polymer derivatives of insulin improve the intestinal absorption of insulin: carbamylation of the amino groups of Phe^{B1} and Lys^{B29} with long-chain fatty acid polymers yield compounds which provide some degree of hypoglycemic activity. This derivatization increases the stability of insulin in intestinal mucosa and its absorption from the small intestine.

[0044] While the ensuing description is primarily and illustratively directed to the use of insulin as a peptide component in various compositions and formulations of the invention, it will be appreciated that the utility of the invention is not thus limited, but rather extends to any peptide species which are covalently or associatively conjugatable in the manner of the invention, including, but not limited to, the following peptide species: calcitonin, ACTH, glucagon, somatostatin, somatotropin, somatomedin, parathyroid hormone, erythropoietin, hypothalmic releasing factors, prolactin, thyroid stimulating hormone, endorphins, antibodies, hemoglobin, soluble CD-4, clotting factors, tissue plasminogen activator, enkephalins, vasopressin, non-naturally occurring opioids, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminease, adenosine deaminase ribonuclease, trypsin, chemotrypsin, and papain, alkaline phosphatase, and other suitable enzymes, hormones, proteins, polypeptides, enzyme-protein conjugates, antibody-hapten conjugates, viral epitopes, etc.

[0045] One objective of the present invention is to provide suitable polymers for conjugation with peptides so as to obtain the desirable characteristics enumerated above. Another objective is to utilize such modified peptides for sustained *in vivo* delivery of the peptide. Yet another objective is to use the technology to deliver peptides orally in their active form.

[0046] A further objective is to employ associatively conjugated peptides for use in immunoassay, diagnostic, and other non-therapeutic (e.g., *in vitro*) applications. Still another objective of the present invention is to provide stabilizingly conjugated peptide compositions, including covalently bonded compositions variously suitable for *in vivo* as well as non-*in vivo* applications, and to alternatively provide non-covalent, associatively conjugated peptide compositions variously suitable for *in vivo* as well as non-*in vivo* applications.

[0047] A single polymer molecule may be employed for conjugation with a plurality of peptide species, and it may also be advantageous in the broad practice of the invention to utilize a variety of polymers as conjugating agents for a given peptide; combinations of such approaches may also be employed. Further, stabilizingly conjugated peptide compositions may find utility in both *in vivo* as well as non-*in vivo* applications. Additionally, it will be recognized that the conjugating polymer(s) may utilize any other groups, moieties, or other conjugated species, as appropriate to the end use application. By way of example, it may be useful in some applications to covalently bond to the polymer a functional moiety imparting UV-degradation resistance, or antioxidation, or other properties or characteristics to the polymer. As a further example, it may be advantageous in some applications to functionalize the polymer to render same reactive or cross-linkable in character, to enhance various properties or characterisics of the overall conjugated material. Accordingly, the polymer may contain any functionality, repeating groups, linkages, or other constitutent structures which do not preclude the efficacy of the conjugated composition for its intended purpose. Other objectives and advantages of the present invention will be more fully apparent from the ensuing disclosure and appended claims.

[0048] Illustrative polymers that may usefully be employed achieve these desirable characteristics are described herein below in an exemplary reaction scheme. In covalently bonded peptide applications, the polymers may be functionalized and then coupled to free amino acid(s) of the peptide(s) to form labile bonds which permit retention of activity with the labile bonds intact. Removal of the bond by chemical hydrolysis and proteolysis then enhances the peptidal activity.

[0049] The polymers utilized in the invention may suitably incorporate in their molecules constituents such as edible fatty acids (lipophilic end), polyethylene glycols (water soluble end), acceptable sugar moieties (receptor interacting end), and spacers for peptide attachment. Among the polymers of choice, polysorbates are particularly preferred and are chosen to illustrate various embodiments of the invention in the ensuing discussion herein. The scope of this invention is of course not limited to polysorbates, and various other polymers incorporating above-described moieties may usefully be employed in the broad practice of this invention. Sometimes it may be desirable to eliminate one of such moieties and to retain others in the polymer structure, without loss of objectives. When it is desirable to do so, the preferred moieties to eliminate without losing the objectives and benefits of the invention are the sugar and/or the spacer moieties.

[0050] It is preferred to operate with polymers whose molecular weights fall between 500 and 10,000 daltons.

[0051] In the practice of the present invention, polyalkylene glycol residues of C_2-C_4 alkyl polyalkylene glycols, preferably polyethylene glycol (PEG), are advantageously incorporated in the polymer systems of interest.

[0052] The presence of these PEG residues will impart hydrophilic properties to the polymer and to the corresponding

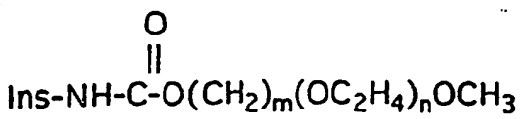
polymer-peptide conjugates. The invention therefore contemplates polymer-peptide products in which the peptide, e. g., insulin, is conjugated with either the hydrophilic or hydrophobic residue of the polymer. The fatty acid portion of the polymer is provided to associate with the hydrophobic domain of the peptide and thus prevent aggregation in solution. The resulting polymer-peptide conjugates thus will be: stabilized (to chemical and enzymatic hydrolysis); water-soluble, due to the PEG residue; and, by virtue of the fatty acid-hydrophobic domain interactions; not prone to aggregation.

[0053] Polyalkylene glycol derivatization has a number of advantageous properties in the formulation of polymer-peptide conjugates in the practice of the present invention, as associated with the following properties of polyalkylene glycol derivatives: improvement of aqueous solubility, while at the same time eliciting no antigenic or immunogenic response; high degrees of biacompatibiiity; absence of *in vivo* biodegradation of the polyalkylene glycol derivatives; and ease of excretion by living organisms.

[0054] The polymers employed In the practice of the present invention thus comprise lipophilic and hydrophilic moieties, rendering the resulting polymer-peptide conjugate highly effective (bioactive) in oral as well as parenteral and other modes of physiological administration, and highly effective in non-physiological applications.

[0055] Set out below as illustrative examples of polymer-peptide conjugates of the present invention is the formulae of a covalently bonded conjugate denoted for ease of subsequent reference as Conjugate 3, wherein "Ins" is insulin, and specific values of m, n, w, x, and y will be described in the ensuing discussion.

Conjugate 3:



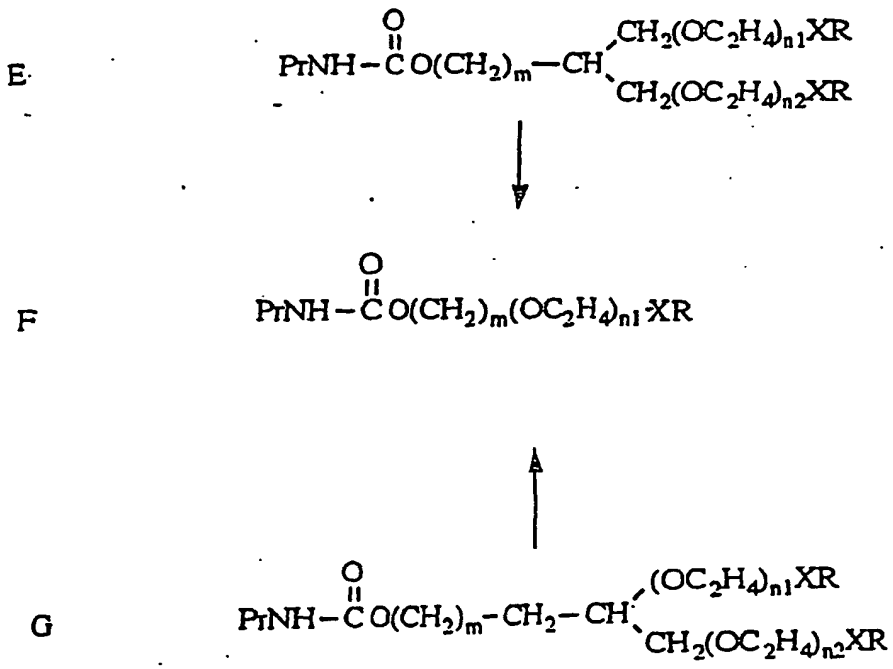
[0056] In Conjugate 3 the lipophilic fatty acid residue is closest to the point of attachment to insulin and the hydrophilic PEG region is distant from the point of attachment, which is through a carbamate bond.

[0057] In Conjugate 3 the point of attachment of the carbamate bond between the polymers is preferably the amine function of Gly^{A1}. It is possible, as mentioned, that more than one polymer unit may be attached to each molecule of peptide. For example, if a second polymer is attached to insulin, the point of attachment preferably is through the amine function of Phe^{B1}. In theory at least, a third polymer could be attached to the amine function of Lys^{B29}. Experience has shown however that two polymer attachments per Insulin molecule is the highest practical degree of derivatization reasonably obtainable.

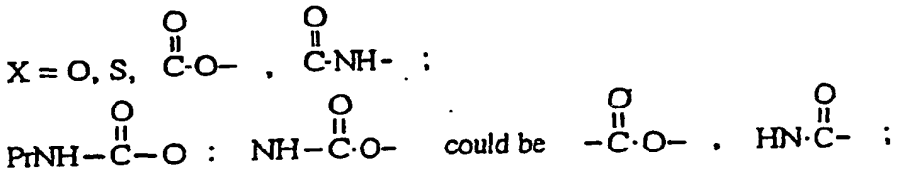
[0058] In the general practice of the invention, various methods of coupling the polymers to the peptide are available and are discussed more fully hereinafter. In working with proteins and polypeptides, it should be realized that certain residue groups in the peptide are important in their overall biological integrity. It is important to choose a suitable coupling agent that does not unduly interfere with such residues. In some instances, it may be difficult to avoid coupling and therefore masking the activity of these important residues, but some activity may be traded for increased stability while maintaining the endowed beneficial properties. In *in vivo* applications, for example, frequency of dosing may thus be reduced, resulting in reduced costs and increased patient compliance.

[0059] The polymers utilized in protein/peptide conjugation in accordance with the invention are designed to incorporate good physical characteristics that enable them to achieve the desired objectives. Absorption enhancers, while enabling penetration of peptides through the cell membrane, do not improve the stability characteristics of the peptides, and *in vivo* applications may therefore utilize the polymer-peptide conjugates of the invention in formulations devoid of such penetration enhancers. One aspect of the present invention therefore relates to the incorporation of fatty acid derivatives within the polymer, to mimic penetration enhancers.

[0060] In the covalently conjugated polymer-peptide conjugates of the present invention, the peptide may be covalently attached to the water-soluble polymer by means of a labile chemical bond. This covalent bond between the peptide and the polymer may be cleaved by chemical or enzymatic reaction. The polymer-peptide product retains an acceptable amount of activity; full activity of the component peptide is realized when the polymer is completely cleaved from the peptide. Concurrently, portions of polyethylene glycol are present in the conjugating polymer to endow the polymer-peptide with high aqueous solubility and prolonged blood circulation capability. The modifications described above confer improved solubility, stability, and membrane affinity properties on the peptide. As a result of these improved characteristics the invention contemplates parenteral and oral delivery of both the active polymer-peptide species and, following hydrolytic cleavage, bioavailability of the peptide *per se, in vivo* applications.

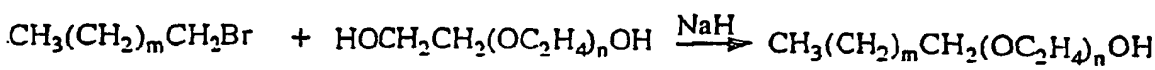


Pr = Peptides. Proteins, Protein Drugs ;
R = Alkyl, C_5 to C_{18} ;
n = 5 to 120 ;
m = 2 to 15 ;



[0061] In the synthesis of Polymer G, it is desirable to protect the hydroxyl moieties on the first and second carbon of glycerol, e.g. solketal the protected glycerol is first reacted with esters of fatty acids which have been halogenated at the terminal carbon of the acid. The remaining hydroxyl group is converted to the sodium salt in an inert solvent and reacted with halogenated or tosylated polyethylene glycol in which one end of the polyethylene glycol has been protected as an ester. The glycerol protection is removed and the resulting two free hydroxyl groups are converted to the corresponding sodium salts. These salts are reacted in inert solvent with polyethylene glycol which has been partially derivatized with fatty acids. Reaction takes place after the free hydroxyl is converted to the tosylate or bromide.

[0062] In one instance of this embodiment the polyethylene glycol with two terminal free hydroxyl groups is treated with sodium hydride in inert solvent. One equivalent weight of a primary bromide derivative of a fatty acid-like moiety is added to the polyethylene glycol solvent mixture. The desired product is extracted in inert solvent and purified by column chromatography if necessary.



(Formula 9)

[0063] Where it is desired to form a carbamate bond with the polypeptide, the carboxyl or ester is converted to a hydroxyl group by a chemical reduction method known in the art.

(Formula 13)

[0064] The present invention provides conjugates of biocompatible polymers with as biologically active macromolecules, diagnostic reagents, etc., which may for example consist of peptides, proteins, enzymes, growth hormones, growth factors and the like. Such macromolecular compounds may be built with alpha-amino acids joined in an amide linkage to form peptide oligomers and polymers. Depending on the functions of these substances, the peptide components can be proteins, enzymes, growth hormones, etc. For the purpose of brevity, these substances are collectively referred to here as peptides and are designated as Pr. In all cases, biologically active peptides contain free amino or carboxyl groups. Linkage between the polymer and peptides is generally effected through free amino or carboxyl groups.

[0065] The peptides chosen for the purposes of illustration herein are of particular interest in the fields of medicine, agriculture, science, and domestic, as well as industrial applications. They may be enzymes utilized in replacement therapy; hormones for promoting growth in animals, or cell growth in cell culture; or active proteinaceous substances used in various applications, e.g., biotechnology and biological and medical diagnostics. Among the enzymes that can be mentioned are superoxide dismutase, interferon, asparaginease, glutamase, arginase, arginine deaminase, adenosine deaminase ribonuclease, trypsin, chromotrypsin, and papin. Among the peptide hormones that can be mentioned are insulin, calcitonin, ACTH, glucagon, somatosin, somatropin, somatomedin, parathyroid hormone, erthyropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, and vasopressin.

[0066] In general, various techniques may be advantageously employed to improve the stability characteristics of the polymer-peptide conjugates of the present invention, including: the functionalization of the polymer with groups of superior hydrolysis resistance, e.g., the previously illustrated conversion of ester groups to ether groups; modifying the lipophilic/hydrophilic balance of the conjugating polymer, as appropriate to the peptide being stabilized by the polymer; and tailoring the molecular weight of the polymer to the appropriate level for the molecular weight of the peptide being stabilized by the polymer.

[0067] The unique property of polyalkylene glycol-derived polymers of value for therapeutic applications of the present invention is general biocompatibility. The polymers have various water solubility properties and are not toxic. They are non-antigenic, non-immunogenic and do not interfere with biological activities of enzymes. They have long circulation in the blood and are easily excreted from living organisms.

[0068] The products of the present invention have been found useful in sustaining the biological activity of peptides and may for example be prepared for therapeutic administration by dissolving in water or acceptable liquid medium. Administration is by either the parenteral or oral route. Fine colloidal suspensions may be prepared for parenteral administration to produce a depot effect, or by the oral route.

[0069] In the dry, lyophilized state, the peptide-polymer conjugates of the present invention have good storage stability; solution formulations of the conjugates of the present invention are likewise characterized by good storage stability.

[0070] The therapeutic polymer-peptide conjugates of the present invention may be utilized for the prophylaxis or treatment of any condition or disease state for which the peptide consituent is efficacious.

[0071] In addition, the polymer-peptide conjugates of the present invention may be utilized in diagnosis of constituents, conditions, or disease states in biological systems or specimens, as well as for diagnosis purposes in non-physiological systems.

[0072] Further, the polymer-peptide conjugates of the invention may have application in prophylaxis or treatment of condition(s) or disease state(s) in plant systems. By way of example, the peptide component of the conjugate may have insecticidal, herbicidal, fungicidal, and/or pesticidal efficacy amenable to usage in various plant systems.

[0073] Still further, the peptide component of the conjugates of the present invention may be antibodies or alternatively antigenic in character, for diagnostic, immunological, and/or assay purposes.

[0074] In therapeutic usage, the present invention contemplates a method of treating an animal subject having or latently susceptible to such condition(s) or disease state(s) and in need of such treatment, comprising administering to such animal an effective amount of a polymer-peptide conjugate of the present invention which is therapeutically effective for said condition or disease state.

[0075] Subjects to be treated by the polymer-peptide conjugates of the present invention include both human and non-human animal (e.g., bird, dog, cat, cow, horse) subjects, and preferably are mammalian subjects, and most pref-

erably human subjects.

[0076] Depending on the specific condition or disease state to be combatted, animal subjects may be administered polymer-peptide conjugates of the invention at any suitable therapeutically effective and safe dosage, as may readily be determined within the skill of the art, and without undue experimentation.

[0077] In general, suitable doses of the formula (1) compounds for achievement of therapeutic benefit, will be in the range of 1 microgram (μg) to 100 milligrams (mg) per kilogram body weight of the recipient per day, preferably in the range of 10 μg to 50 mg per kilogram body weight per day and most preferably in the range of 10 μg to 50 mg per kilogram body weight per day. The desired dose is preferably presented as two, three, four, five, six, or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing from 10 μg to 1000 mg, preferably from 50 μg to 500 mg, and most preferably from 50 μg to 250 mg of active ingredient per unit dosage form. Alternatively, if the condition of the recipient so requires, the doses may be administered as a continuous infusion.

[0078] The mode of administration and dosage forms will of course affect the therapeutic amounts of the compounds which are desirable and efficacious for the given treatment application.

[0079] For example, orally administered dosages are typically at least twice, e.g., 2-10 times, the dosage levels used in parenteral administration methods, for the same active ingredient.

[0080] The polymer-peptide conjugates of the invention may be administered per se as well as in the form of pharmaceutically acceptable esters, salts, and other physiologically functional derivatives thereof.

[0081] The present invention also contemplates pharmaceutical formulations, both for veterinary and for human medical use, which comprise as the active agent one or more polymer-peptide conjugate(s) of the invention.

[0082] In such pharmaceutical and medicament formulations, the active agent preferably is utilized together with one or more pharmaceutically acceptable carrier(s) therefor and optionally any other therapeutic ingredients. The carrier (s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof. The active agent is provided in an amount effective to achieve the desired pharmacological effect, as described above, and in a quantity appropriate to achieve the desired daily dose.

[0083] The formulations include those suitable for parenteral as well as non-parenteral administration, and specific administration modalities include oral, rectal, buccal, topical, nasal, ophthalmic, subcutaneous, intramuscular, intravenous, transdermal, intrathecal, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic,vaginal, and intra-uterine administration. Formulations suitable for oral and parenteral administration are preferred.

[0084] When the active agent is utilized in a formulation comprising a liquid solution, the formulation advantageously may be administered orally or parenterally. When the active agent is employed in a liquid suspension formulation or as a powder in a biocompatible carrier formulation, the formulation may be advantageously administered orally, rectally, or bronchially.

[0085] When the active agent is utilized directly in the form of a powdered solid, the active agent may advantageously be administered orally. Alternatively, it may be administered bronchially, via nebulization of the powder in a carrier gas, to form a gaseous dispersion of the powder which is inspired by the patient from a breathing circuit comprising a suitable nebulizer device.

[0086] The formulations comprising the active agent of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the active compound(s) into association with a carrier which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the active compound (s) into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation.

[0087] Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the active ingredient as a powder or granules; or a suspension in an aqueous liquor or a non-aqueous liquid, such as a syrup, an elixir, an emulsion, or a draught.

[0088] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, with the active compound being in a free-flowing form such as a powder or granules which optionally is mixed with a binder, disintegrant, lubricant, inert diluent, surface active agent, or discharging agent. Molded tablets comprised of a mixture of the powdered active compound with a suitable carrier may be made by molding in a suitable machine.

[0089] A syrup may be made by adding the active compound to a concentrated aqueous solution of a sugar, for example sucrose, to which may also be added any accessory ingredient(s). Such accessory ingredient(s) may include flavorings, suitable preservative, agents to retard crystallization of the sugar, and agents to increase the solubility of any other ingredient, such as a polyhydroxy alcohol, for example glycerol or sorbitol.

[0090] Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active compound, which preferably is isotonic with the blood of the recipient (e.g., physiological saline solution).

Such formulations may include suspending agents and thickening agents or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose form.

[0091] Nasal spray formulations comprise purified aqueous solutions of the active compounds with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucus membranes.

[0092] Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, hydrogenated fats, or hydrogenated fatty carboxylic acid.

[0093] Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

[0094] Topical formulations comprise the active compound dissolved or suspended in one or more media, such as mineral oil, petroleum, polyhydroxy alcohols, or other bases used for topical pharmaceutical formulations.

[0095] In addition to the aforementioned ingredients, the formulations of this invention may further include one or more accessory ingredient(s) selected from diluents, buffers, flavoring agents, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants), and the like.

[0096] In non-therapeutic applications of the present invention, the polymer-peptide conjugate may utilize a covalently bonded or alternatively non-covalent bonding relation between the peptide and polymer components. In addition, associatively related peptide and polymer components may be utilized in administration of therapeutic peptide agents, by appropriate administration methods such as those illustratively described hereinabove in connection with illustratively discussion of covalently bonded polymer-peptide conjugates of the invention.

[0097] In such non-therapeutic, associatively related peptide-polymer compositions, the peptide and polymer components may be initially formulated together to provide an enhanced stability and degradation resistance; altematively, these components may for example be separate parts of a multipart composition which is mixed at time of use, and which in the absence of associative bonding between the polymer and peptide in the resulting mixture would be susceptible to quick decay or other degradative modality. Regardless of the form of the associatively related peptide and polymer composition, the present invention contemplates a relational association which enhances some characteristic or aspect of the peptide or otherwise enhances the utility of same, relatively to the peptide component in the absence of such associative polymer.

[0098] Accordingly, the present invention contemplates the provision of suitable polymers for *in vitro* stabilization of peptides in solution, as a preferred illustrative application of non-therapeutic application. The polymers may be employed for example to increase the thermal stability and enzymic degradation resistance of the peptide. Enhancement of the thermal stability characteristic of the pepide via conjugation in the manner of the present invention provides a means of improving shelf life, room temperature stability, and robustness of diagnostic and research reagents and kits, e.g., immunoassay kits. By way of specific example, alkaline phosphatase may be covalently or associatively coupled to a suitable polymer in accordance with the invention, to impart stability to such phosphatase when used as a reagent in kits for colorimetric detection of antibody or antigen in biological fluids.

[0099] The following Examples are provided to illustrate the present invention, and should not be construed as limiting thereof.

Reference Example I

Reference Conjugate 1

Polysorbate trioleate p-nitrophenyl carbonate

[0100] To a solution of p-nitrophenylchloroformate (0.8g. 4 mmole) in 50 mL of anhydrous acetonitrile is added dry polysorbate trioleate (7g, 4 mmole) followed by dimethylaminopyridine (0.5g, 4 mmole). The reaction mixture is stirred at room temperature for 24 hours Solvent is removed under reduced pressure and the resultant precipitate is diluted with dry benzene and filtered through Celite. The residue is refrigerated overnight in dry benzene and the additional precipitate is removed by filtration. Solvent is removed under reduced pressure and residual benzene is removed by evacuation at low pressure to yield 6.4g of polysorbate trioleate p-nitrophenyl carbonate.

Coupling of insulin with activated polymer

[0101] To a solution of activated polysorbate trioleate p-nitrophenylchloroformate (1g) in distilled water is added a solution of bovine insulin (50 mg) in 0.1 M pH 8.8 phosphate buffer. pH is maintained by addition of 1N NaOH as necessary. The reaction mixture is stirred at room temperature for 2.5 h. After this time the mixture is subjected to gel filtration chromatography using Sephadex G-75. Purification by elution with 0.1M pH 7.0 phosphate buffer and collection

of fractions with an automated fraction collector yields reference Conjugate 1. The polymer content is determined by trinitrobenzenesulfonic acid (TNBS) assay and the protein concentration by Biuret Method. A molar ratio of polymer to insulin is determined to be 1:1.

Example I

Conjugate I

[0102] The terminal hydroxyl group of polyethylene glycol monostearate is activated by reaction with p-nitrophenyl chloroformate as described above. To a solution of the activated polymer (1g) in distilled water is added bovine insulin (80mg) dissolved in 0.1 M phosphate buffer, at pH 8.8. The pH is maintained by careful adjustment with 1N NaOH. After stirring for 3 hours, the reaction mixture is quenched with excess glycine and subjected to gel filtration chromatography using Sephadex G-75. Insulin/polymer conjugate is collected and lyophilized. Protein content is determined by Biuret assay, giving a quantitative yield.

Example II

Conjugate 2

Tetrahydro-2-(12-bromododecanoxy)-2H pyran

[0103] To a solution of 12-bromo-1-dodecanol (1 mole) in dichloromethane containing pyridinium p-toluenesulfonate (P-TSA) is added dihydropyran (2 moles). The reaction mixture is stirred for 24 hours and then washed twice with water and dried over anhydrous $MgSO_4$. The dichloromethane is removed under reduced pressure. If necessary the resulting product is purified by chromatography on silica gel.

Coupling of polyethylene glycol to the terahydropyran derivative

[0104] The tetrahydropyran derivative described above, dissolved in dry benzene, is added to a solution of polyethylene glycol (1 mole) in dry benzene containing NaH (1 mole). The reaction mixture is stirred at room temperature for 24 hours. After that time the mixture is eluted through a silica gel column with benzene. Additional purification by column chromatography, if necessary, is performed. The protective tetrahydropyran group is removed by treatment with p-TSA at room temperature. The final product is purified, if necessary, by column chromatography. The hydroxyl group of the polymer is activated by reaction with p-nitrophenylchloroformate as described hereinabove. Conjugation with insulin is carried out as described for reference Conjugate 1.

Example III

[0105] Comparative studies using bovine insulin were conducted on polymer-insulin conjugates and on native insulin to determine their relative stability and activity in animal models. In the animal studies, the efficacy of the polymer-insulin in lowering the blood level was compared to that of native insulin. Female and male albino mice averaging 25 g in weight were fasted overnight and used in groups of five for each treatment conducted in several phases over a period of two days.

[0106] Each test animal received a single dose of either native insulin (Group 1, 100 μg/kg, subcutaneously); native insulin (Group 2, 1.5 mg/kg, orally by gavage); reference Conjugate 1) Group 3, 100 μg/kg, orally); or reference Conjugate 1 (Group 4, 100 μg/kg, subcutaneously) at time 0. An additional group (Group 5) received no insulin of any kind but was challenged with glucose 30 minutes before scheduled sampling times. Animals were fasted overnight before treatment and for the duration of the study. All test materials were prepared in phosphate buffered saline, pH 7.4. Thirty minutes before scheduled sampling times of 0.5, 1, 2, 4, 8 and 24 hours following treatment with insulin, animals were challenged with a bolus dose of glucose (5g/kg, as a 50% solution, given orally), so that each animal received only one dose of insulin or reference Conjugate 1 and one glucose challenge. At the scheduled sample time blood was collected from the tail vein and immediately analyzed for glucose content using a One Touch Digital Glucose Meter (Life Scan). The results of the test are shown in Figure 1, for Groups 1-5.

[0107] Blood glucose levels for Group 1 animals (native insulin, subcutaneous) were approximately 30% of control (Group 5, untreated) animals at the 30 minute time point. This hypoglycemic effect lasted only 3.5 hours in Group 1 animals. Native insulin administered orally (Group 2) lowered blood glucose levels to a maximum of 60% of control, this maximum response occurring 30 minutes after treatment with the insulin. In contrast the glucose levels in animals in Group 3 (reference Conjugate 1, 100 μg/kg, p.o.) were lowered with an apparent delayed onset of hypoglycemic

activity. The hypoglycemic activity in Group 3 animals was greater than that in Group 2 animals even though the dose of insulin administered to Group 3 was only one fifteenth of that given to Group 2. At all time points after 3 hours glucose levels were lower for Group 3 animals than for any other treatment group, the largest difference being at the four to eight hour sampling points. Glucose levels in Group 4 animals (reference Conjugate 1, 100 μg/kg, s.c.) followed the same course as those for Group 1 animals for the first four hours of the study. After four hours Group2 glucose levels remained above control (untreated, Group 5) levels whereas Group 4 glucose levels dropped, at eight hours, to 62% of Group 5 levels, and remained below Group 5 levels.

Example IV

[0108] An insulin efficacy study was conducted on male and female albino mice using as test materials insulin in unconjugated form, and reference Conjugate 1. One objective of this study was to determine whether insulin in the form of reference Conjugate 1 is capable of acting on blood glucose levels in the same way as insulin when administered subcutaneously. A second objective was to determine whether the insulin complex of reference Conjugate 1, unlike free insulin, is capable of acting to decrease the blood glucose level when administered orally. The results are shown in Figure 2, wherein "Insulin Complex" denotes reference Conjugate 1.

[0109] Baseline blood samples were obtained for serum glucose analysis from 10 fasted untreated albino mice (5 males and 5 females); baseline values in Figure 2 are denoted by the symbol "O". Three additional groups (five males and five females each) were fasted overnight and loaded with glucose alone orally by gavage (5 g/kg body weight). Ten animals were sacrificed at each of three time periods to obtain blood samples for glucose analysis: 30, 60 and 120 minutes after dosing. A commerial insulin and reference Conjugate 1 were each administered both orally (p.o) and parenterally (s.c.) to groups of fasted mice (five males and five females, for sacrifice and blood analysis at each of the three time periods), to provide different treatment regimens. The treatment, administration routes, and symbols shown for results in Figure 2 included: (i) glucose (5g/kg p.o.), symbol: "●"; (ii) insulin (100μg/kg, s.c.) and glucose (5g/kg p. o.), symbol: "▽"; (iii) insulin (1.5mg/kg, p.o.) and glucose (5g/kg p.o.), symbol: "▽"; (iv) reference Conjugate 1 (100μg/kg, s.c.) and glucose (5g/kg p.o.), symbol "□"; (v) reference Conjugate 1 (250μg/kg, s.c.) and glucose (5g/kg p.o.), symbol: "□"; (vi) reference Conjugate 1 (1.5mg/kg, s.c.) and glucose (5g/kg p.o.), symbol: "Δ". In these tests of reference Conjugate 1, the concentration of the protein in the administered solution was 0.1 mg protein/ml solution; for comparison purposes, a modified covalently bonded insulin-polymer conjugate, having a protein concentration in the administered solution of 0.78mg protein/ml solution, was included, (vii) modified reference Conjugate 1 (100μg/kg, s.c.) and glucose (5g/kg p.o.), symbol: "Δ".

[0110] The insulin was administered 15 minutes prior to glucose loading.

[0111] Glucose was administered orally by gavage to all but the baseline group of animals at a dose of 5g/kg (10mg/kg of a 50% w/v solution in normal saline). When insulin was administered orally by gavage, it was given at a dose of 1.5mg/kg (18.85 ml/kg of a 0.008% w/v solution in normal saline). When insulin was administered subcutaneously, it was given at a dose of 100μg/kg (2.5 ml/kg of a 0.004% w/v solution in normal saline). When the reference Conjugate 1 polymer-insulin complex was administered orally by gavage, it was given at a dose of 1.56mg/kg (2.0 ml/kg of the undiluted test material). When the reference Conjugate 1 polymer-insulin complex was administered subcutaneously, it was given at a dose of 100μg/kg (1.28 ml/kg of a 1:10 dilution of the 0.78 mg/ml solution received) or 250μg/kg (3.20 ml/kg of a 1:10 dilution of the solution received). The modified reference Conjugate 1 contained 0.1 ml insulin/ml and was dosed at a rate of 1.0 ml/kg to obtain a 100μg/kg dose.

[0112] Glucose was measured using the Gemini Centrifugal Analyzer and purchased glucose reagent kits. The assay was a coupled enzymatic assay based on the reaction of glucose and ATP catalyzed by hexokinase, coupled with the glucose-6-phosphate dehydrogenase reaction, yielding NADH. Duplicate samples were analyzed and the mean value reported. Dilution (1:2 or 1:4) of some serum samples was necessary in order to determine the very high glucose concentration present in certain samples.

[0113] After glucose loading, mean serum glucose rose to a high level at 30 minutes, declined at 60 minutes, and was below baseline at 120 minutes. If commercial insulin was administered subcutaneously (100μg/kg body weight, it was highly effective in preventing the increase in blood glucose. However, if insulin was given orally (at a high dose of 1.5 mg/kg) there was no effect on the rise of blood glucose. This was expected, since insulin, a protein, is readily hydrolyzed in the digestive tract and is not absorbed intact into the bloodstream.

[0114] When reference Conjugate 1 was given subcutaneously at either 100 or 250 μg/kg dosage, it was highly effective in restricting the rise in blood glucose after glucose loading. Mean serum glucose values were significantly lower after the 100μg/kg dose of reference Conjugate 1 at both 30 and 60 minutes than they were after 100μg/kg of free insulin. Mean serum glucose at 250μg/kg of reference Conjugate 1 was lower, though not significantly, at 30 minutes, significantly lower at 60 minutes and at 120 minutes was returned to the baseline. With both free insulin at 100μg/kg and reference Conjugate 1 at 100μg/kg, the glucose level remained below baseline at 120 minutes.

[0115] The modified reference Conjugate 1 administered at 100μg/kg produced a significant reduction in blood glu-

cose at 30 minutes.

reference Example II

Preparation of Para-Nitrophenyl Carbonate of Polysorbate Monopalmitate

[0116] Polysorbate monopalmitate is first dried by the azeotropic method using dry benzene.

[0117] To a solution of the dry polymer (2g, 2 mmole) in 10 ml of dry pyridine is added para-nitrophenylchloroformate (0.6g, 3 mmol). The mixture is stirred at room temperature for 24 hours. The reaction mixture is chilled in ice and diluted with dry benzene and filtered through filter aid. This procedure is repeated and finally the solvent is removed at the rotary evaporator. Traces of solvent are removed in vacuo. The yield of the product is 1.8g.

reference Example III

Preparation of Polysorbate Monopalmitate Conjugate with Insulin

[0118] In accordance with the previously described conjugation reaction procedure of reference Example I but using polysorbate monopalmitate in the amount of 1g and insulin in the amount of 80 mg, with HPLC separation of the reaction product, an insulin-polysorbate monopalmitate covalently bonded conjugate is obtained.

EXAMPLE V

Preparation of Enzyme-polymer Conjugates

[0119] Coupling of alkaline phosphatase (AP) to polymer was carried out using the same procedure as described for reference Conjugate 1 in reference Example I. In addition, to determine whether a high or low ratio of polymer to protein would be more advantageous, conjugates were prepared using 140 moles of polymer/mole of enzyme and 14 moles of polymer/mole of enzyme. The number of polymer groups per molecule of conjugated AP are 30 and 5, respectively, for the high and low ratios of polymer.

[0120] The following procedure was employed to obtain about 5 groups/molecule of alkaline phosphatase: 4.1 mg (salt free) was dissolved in 0.05M. sodium bicarbonate. To this solution was added activated polymer (0.75 mg) in water/dimethyl-sulfoxide and the solution was stirred for 3 to 12 hours at room temperature. The resullting reaction mixture was dialyzed against a salt solution (0.3N NaCl) in dialysis tubing (MW cutoff 12,000-14,000) over 12 hours with 4 to 6 changes of dialysis solution. The same procedure was used for the high ratio. Total protein concentration of dialyzed material was determined by Biuret method.

Activity Measurement and Stability Study

[0121] The phophatase assay was performed according to the method of A. Voller et al, Bulletin WHO, 53, 55 (1976). An aliquot (50 microliter) was added to microwells and mixed with 200 microliter of substrate solution (10g/L, 4-nitrophenylphosphate in 20% ethanolamine buffer, pH 9.3) and incubated at room temperature for 45 minutes. The reaction was stopped by 50 microliter of 3M NaOH. The absorbance was measured at 405 nm in a micro plate reader.

[0122] Phosphatase activity was compared with that of native enzyme under various conditions.

[0123] Dilute solutions containing similar concentrations of alkaline phosphatase and alkaline phosphatase-polymer conjugates were stored at various temperatures. The enzymatic activity was tested periodically. The two polymers tested at 5°C, 15°C, 35°C and 55°C were compared to the control alkaline phosphatase stored at 5°C.

[0124] As can be seen from Table A, the initial enzymatic activity of both polymers was about three-fold higher than the control. Both polymer-enzyme conjugates had enhanced thermal stability over the native enzyme. This is especially evident for the conjugate characterized by the higher ratio of polymer to enzyme.

Table A

		DAY					
TEMP, °C		O	2	3	4	5	6
AP/HIGH	5	399	360	321	371	343	337
	15		158	115	126	24	184
	35		132	112	135	138	123
	55		36	25	10	14	
AP/LOW							
	5	324	252	210	220	162	159
	15		83	47	40	38	51
	35		61	36	35	33	32
	55		17	6	2	2	
AP/CONTROL	5	100	100	100	100	100	100
	15		89	74	43	36	28
	35		53	48	21	20	20
	55		10	2	1	2	

EXAMPLE VI

Conjugate 1A

[0125] To a solution of insulin (50mg) in 0.05 M sodium bicarbonate buffer of pH 9.2 is added a solution of activated polymer (1g) in water/dimethylsulfoxide and stirred for 3 hours, at room temperature. The pH of the mixture is maintained by careful adjustment with 1N NaOH. The reaction mixture then is dialyzed against 0.1M pH 7.0 phosphate buffer. Purified product is lyophilized. Protein content (48mg) is determined by Biuret assay. The number of polymer chains linked to insulin is determined by TNBS assay, giving a ratio of two moles of polymer to one mole of insulin.

reference Example IV

[0126] Calchonin-OT_{50} was synthesized in the following manner. Activated - OT (160 mg) in water (2.0 ml) was added to a stirred solution of salmon calcitonin (5 mg. Becham) in de-ionized water (1.5 ml) and borate buffer, at 5°C. The resulting solution was stirred at 20° C for 1.5 h and the pH of the solution was adjusted to 8.8. The reaction was further stirred for another 0.5 h before the ph was brought to 3.8 with dilute (1M) hydrochloric acid. The reaction mixture was stored at 5° C overnight. The solution was initially dialyzed against PBS buffer (ph 7.5, 2 L) and then against PBS buffer (adjusted to pH 3.6, 4 x 1 L) in a dialysis membrane (MWCO 3500). The dialyzed solution was filtered through a 0.22 microfilter and stored at 5° C. Protein concentration was determined by Biuret and HPLC using native calcitonin as the standard. Purity was analyzed by HPLC on a size exclusion column, using 0.05 M phosphate buffer (adjusted to pH 3.8).

reference Example V

[0127] Calcitonin -- 001 was synthesized in the following manner. Activated 001 (130 mg) In di-methyl sulfoxide (1 ml) was added to a stirred solution of salmon calcitonin (5 mg) in de-ionized water (2 ml) and borate buffer (1.5 ml, 0.1 M, pH 8.8,) at 5° C. The solution was stirred at 20° C for 1.5 h and the pH was adjusted to 8.8 with 1N hydrochloric acid. The reaction was stirred for another one hour before the pH was brought to 3.8 and the reaction mixture stored at 5° C overnight. 4 ml of de-ionized water was added to the reaction mixture and the supernatant was dialyzed initially against phosphate saline buffer (PBS) (pH 7.4, 2L) in the dialysis membrane (MWCO 3500) and then four times against PBS buffer (adjusted to pH 3.6). The dialyzed solution was filtered through a 0.22 μ filter and stored at 5° C. Protein concentration was determined by Biuret and HPLC methods, using native calcitonin as the standard. Purity was analyzed by HPLC using C-8 reverse space with gradient elution of acetonitrile/0.1% TFA (30 to 55% over 25 minutes).

Example VII

[0128] Insulin-OT was prepared in the following manner. 2.0 g of activated OT polymer was weighed in a 250 ml round bottom flask. The OT polymer was dissolved completely in 10 ml of anhydrous DMSO, and 20 ml de-ionized water was added and stirred for 5 minutes. The resulting OT solution was cooled 10° C. 100 mg of insulin (Sigma/Bovine Pancreas/Zn) dissolved in 40 ml of 0.1 M sodium borate buffer (pH 9.3) was added all at once to the OT polymer solution. The reaction mixture turned bright yellow. After 30 minutes, the pH of the solution was adjusted to 8.8 using 2N HCl. The reaction solution was stirred for an additional 1.5 h and the pH was adjusted to 8.4. The reaction mixture was filtered through a 0.8 micrometer filter membrane and dialyzed in PBS buffer (ph 7.4). The dialyzed mixture was filtered through a 0.22 micron filter and concentrated. The concentrated sample was chromatographed using Sephadex G-75 (eluent 0.05 M sodium phosphate buffer). Column: 2.5 cm dia. x 32 cm h. Analysis indicated a yield of insulin conjugate to be quantitative with two moles of polymer per mole insulin.

Example VIII

[0129] 001-insulin was orally administered to treatment groups of glucose challenged cynomolgus monkeys in dosages of 0.5 mg, 2 mg and 5 mg respectively. The area under the curve (AUC) was calculated for each treatment group. Using this measure, lower values represent the greatest efficacy in lowering blood glucose. The AUC results are shown in Fig. 8. These results show that a single orally-administered dose of 001-insulin is effective in protecting against elevation of blood glucose following glucose challenge in cynomolgus monkey. The AUC following oral administration of 001-insulin is less than 1/2 that for untreated animals, demonstrating significant hypoglycemic activity of 001-insulin in cynomolgus monkeys.

Reference Example VI

[0130] Hypocalcemic activity of polymer-calcitonin conjugates given via oral administration was evaluated in a rat hypocalcemic model. Two polymer-calcitonin derivatives (OT-calcitonin and 001-calcitonin) were evaluated in the following manner. Male Sprague-Dawley rats (average weight 54 g) were fasted overnight. Animals were randomly assigned to treatment groups (5 per group) and baseline serum calcium levels were determined for each group. Treatment groups then received a single dose of either unmodified calcitonin, at a dose of 50 mg per kg, OT-calcitonin at a dose of 2.5 mg per kg, and 001-calcitonin at doses of 250 mg per kg, 2.5 mg per kg, and 25 mg per kg, respectively. The doses were given by the oral route. Serum calcium was determined 2 and 4 hours following administration. The results are presented in Fig. 4 as percent of original serum calcium levels over time. The ( ) represents the calcitonin, ( ) represents 001, 250 mg, ◊ represents 001 2.5 μg, Δ represents 001, 25 mg, and ● represents OT, 2.5 μg. The results show significant lowering of serum calcium in a dose-related manner. It appears that the conjugates are active for more than the 4 hour time span initially evaluated. An extended duration of action may correspond to significant bioavailability following oral administration.

Reference Example VII

[0131] The hypocalcemic activity of orally-administered polymer-calcitonin in a rat model was demonstrated in the following manner. Male Sprague-Dawley rats, average weight 54 grams, were fasted overnight. Animals were randomly assigned to treatment groups (4 per group) and baseline serum calcium levels were determined for each group. Control groups received a single dose of unmodified calcitonin (ct) by either the sub-cutaneous (s.c.) route (50 mg per kg) or orally (25 μg per kg). Treatment groups received either OT1-ct or OT2-ct (2.5 μ per kg) by the oral route. Serum calcium was determined 2 and 4 hours following administration. The results are presented in Fig. 5 as percent of original serum calcium levels over time. OT1-ct and 0T2-ct are repeat preparations of the same calcitonin-polymer conjugate, evaluated side by side to demonstrate consistency of response from one batch to the next. The results show significant lowering of serum calcium. It appears that the conjugates are active for more than the four hour timespan initially evaluated.

EXAMPLE IX

[0132] Polymer-insulin is evaluated in a diabetic (BB) rat model in the following manner. The BB rat is a reliable model of human insulin dependent diabetes. Without daily subcutaneous (sc) insulin adminstration BB rats die within two days. In a 14 day study sc insulin was stopped and animals were switched to treatment with orally administered polymer-insulin at low (100 micrograms per kilogram per day), medium (3 milligrams per kilogram per day) and high (6 milligrams per kilogram per day). The results (mean survival time,) are shown in Figure 6. The abrupt switch from

sc to oral administration consitutes particuarlly harsh tests for polymer insulin. The results show that the low dose animals did not receive sufficient insulin to survive; the medium dose group showed some increase in survival time and the high dose group in which some aminals survived for the entire (14 day) duration of the study receiving only orally administered polymer insulin. The polymer insulin was administered by gavage in non-optimized formulation. This study also showed a statistically significant decrease in pm compared to am blood glucose levels and were better controlled if animals received multiple daily administrations instead of a single bolus. Daily oral administration of polymer-insulin to normal (non-diabetic) animals cause significant lower of am blood glucose levels.

EXAMPLE X

[0133] The effect of orally administered 001-insulin in cynomolgous monkeys was shown in the following manner. 6 cynomolgous monkeys (3 male, 3 female, average weight 2.5 kilograms) were fasted overnight. Baseline blood glucose levels were determined before animals received a glucose challenge of 3 grams per kilogram body weight administered by oral gavage as a 50% solution. At various time (0-4 hours) blood glucose levels were reported. Two days later the animals were again fasted and challenged with 3 grams per kilogram body weight glucose. This time the animals received 001-insulin (equivalent to 5 miligrams per kilogram insulin) by the oral route. Blood glucose levels were recorded at various time points. The effect of 2 milligrams per kilogram and 0.5 milligram per kilogram doses of 001-insulin using the same animals and protocol were determined after a two day washout period was allowed between treatment. Results for each treatment (control, 0.5, 2 or 5 milligram per kilogram) were calculated as percent change in blood glucose over time and the results are shown in Figure 7. Wherein the represents glucose represents 5 milligrams, represents 2 milligrams and represents 0.5 milligrams. 2 hours after the glucose challenge, the average increase in blood glucose levels in the treated group is one-third the increase in untreated animals.

EXAMPLE XI

[0134] The chymotrypsin digestion of insulin and OT insulin was measured in the following manner. Chymotrypsin (purified Sigma type 1 S) was incubated at 37°C with either insulin (bovine) or OT-insulin conjugate dissolved in phosphate buffered saline at pH 8. Aliquots were removed periodically and made acidic by addition of one-tenth volume of 2% trifluoroacidic acid (TFA) to arrest the reaction. Samples were analyzed by HPLC and the results are shown in Figure 3 wherein the OT-insulin is represented by a and the insulin is represented by a

Best Mode for Carrying Out the Invention

[0135] Presently preferred conjugation stabilized polypeptide polymer compositions and formulations of the present invention relate to covalently conjugated peptide complexes wherein the peptide is covalently bonded to one or more molecules of the polymer incorporating as a integral part thereof a hydrophillic moiety, e.g, a linear polyalkylene glycol, and wherein said polymer incorporates a lipophilic moiety as an integral part thereof.

[0136] A particularly preferred aspect of the present invention relates to a physiologically active peptide composition comprising a physiologically active peptide covalently coupled with a polymer comprising (i) linear polyalkylene glycol moiety and (ii) a lipophilic moiety, wherein the peptide, linear polyalkylene glycol moiety, and lipophilic moiety are conformationally arranged in relation to one another such that the physiologically active peptide in the physiologically active peptide composition has an enhanced in vivo resistance to enzymatic degradation, relative to the physiologically active peptide alone (i.e., in a conjugated form devoid of the polymer coupled thereto).

Industrial Applicability of the Invention

[0137] The present invention contemplates the use of conjugation stabilized polypeptide compositions herein disclosed, for oral administration dosages for the mediation and treatment of disease states which involve peptide deficiencies.

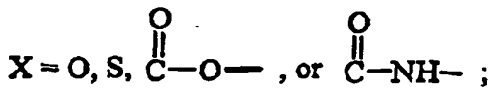
Claims

1. A polymer-peptide conjugate having the following formula:

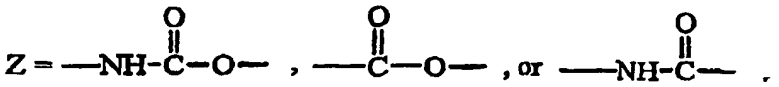
$$Pr—Z—(CH_2)_m(OC_2H_4)_n—XR$$

wherein

Pr=peptides;
R=alkyl;
n=5 to 120;
m=2 to 15;



and



2. A polymer-peptide conjugate according to claim 1 wherein the peptide is insulin.

3. A pharmaceutical composition comprising a polymer-peptide conjugate of claim 1, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

4. Use of a polymer-peptide conjugate of claim 1, or a pharmaceutically acceptable salt thereof, for the manufacture of an oral medicament for the treatment of insulin deficiency.

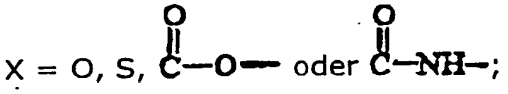
Patentansprüche

1. Polymer-Peptid-Konjugat mit der folgenden Formel:

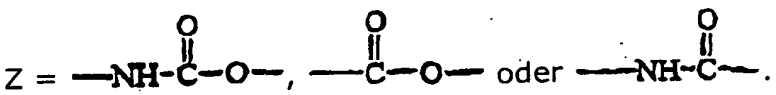
$$Pr\text{-}Z\text{-}(CH_2)_m(OC_2H_4)_n\text{-}XR,$$

wobei

Pr = Peptide;
R = Alkyl;
n = 5 bis 120;
m = 2 bis 15;



und



2. Polymer-Peptid-Konjugat gemäß Anspruch 1, wobei es sich bei dem Peptid um Insulin handelt.

3. Pharmazeutische Zusammensetzung, die ein Polymer-Peptid-Konjugat gemäß Anspruch 1 oder ein pharmazeutisch annehmbares Salz davon sowie einen pharmazeutisch annehmbaren Träger umfasst.

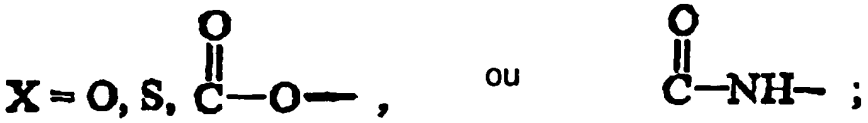
4. Verwendung eines Polymer-Peptid-Konjugats gemäß Anspruch 1 oder eines pharmazeutisch annehmbaren Salzes davon zur Herstellung eines oralen Medikaments für die Behandlung eines Insulinmangels.

Revendications

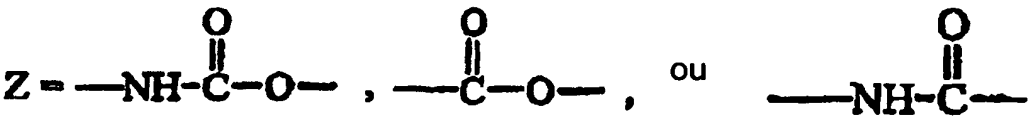
1. Conjugué de polymère - peptide ayant la formule suivante :

$$Pr\text{-}Z\text{-}(CH_2)_m(OC_2H_4)_n\text{-}XR$$

dans laquelle
Pr = peptides ;
R = alkyle ;
n = de 5 à 120 ;
m = de 2 à 15 ;



et



2. Conjugué de polymère - peptide selon la revendication 1, dans lequel le peptide est l'insuline.

3. Composition pharmaceutique comprenant un conjugué de polymère - peptide selon la revendication 1, ou un sel pharmaceutiquement acceptable de celui-ci, et un support pharmaceutiquement acceptable.

4. Utilisation d'un conjugué de polymère - peptide selon la revendication 1, ou d'un sel pharmaceutiquement acceptable de celui-ci, pour la fabrication d'un médicament oral pour le traitement d'une déficience en insuline.

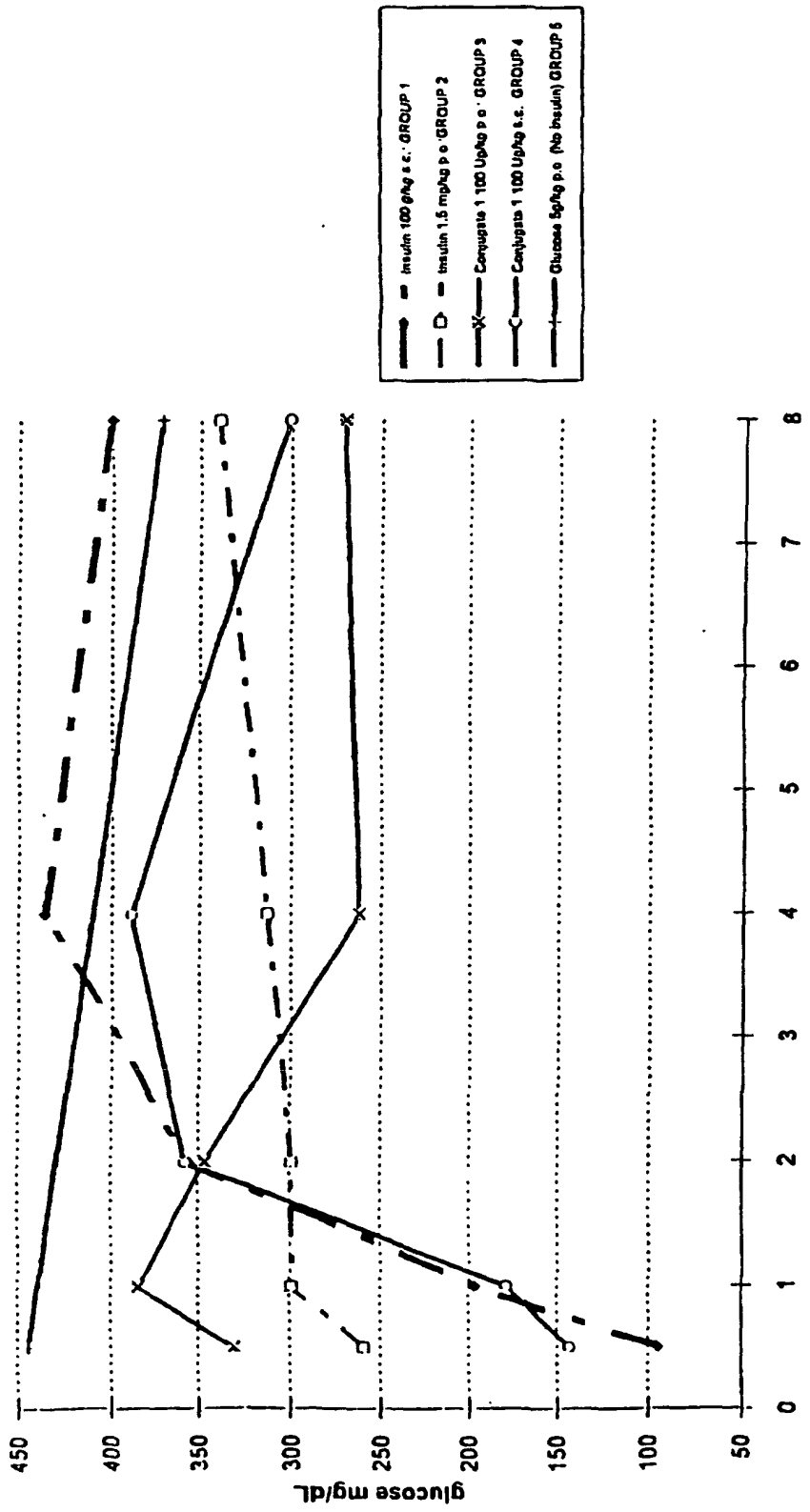

Insulin 100 g/kg s.c. GROUP 1
Insulin 1.5 mg/kg p.o GROUP 2
Conjugate 1 100 Ug/kg p.o GROUP 3
Conjugate 1 100 Ug/kg s.c. GROUP 4
Glucose 5g/kg p.o (No insulin) GROUP 5
glucose mg/dL
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
time, hours

FIG 1

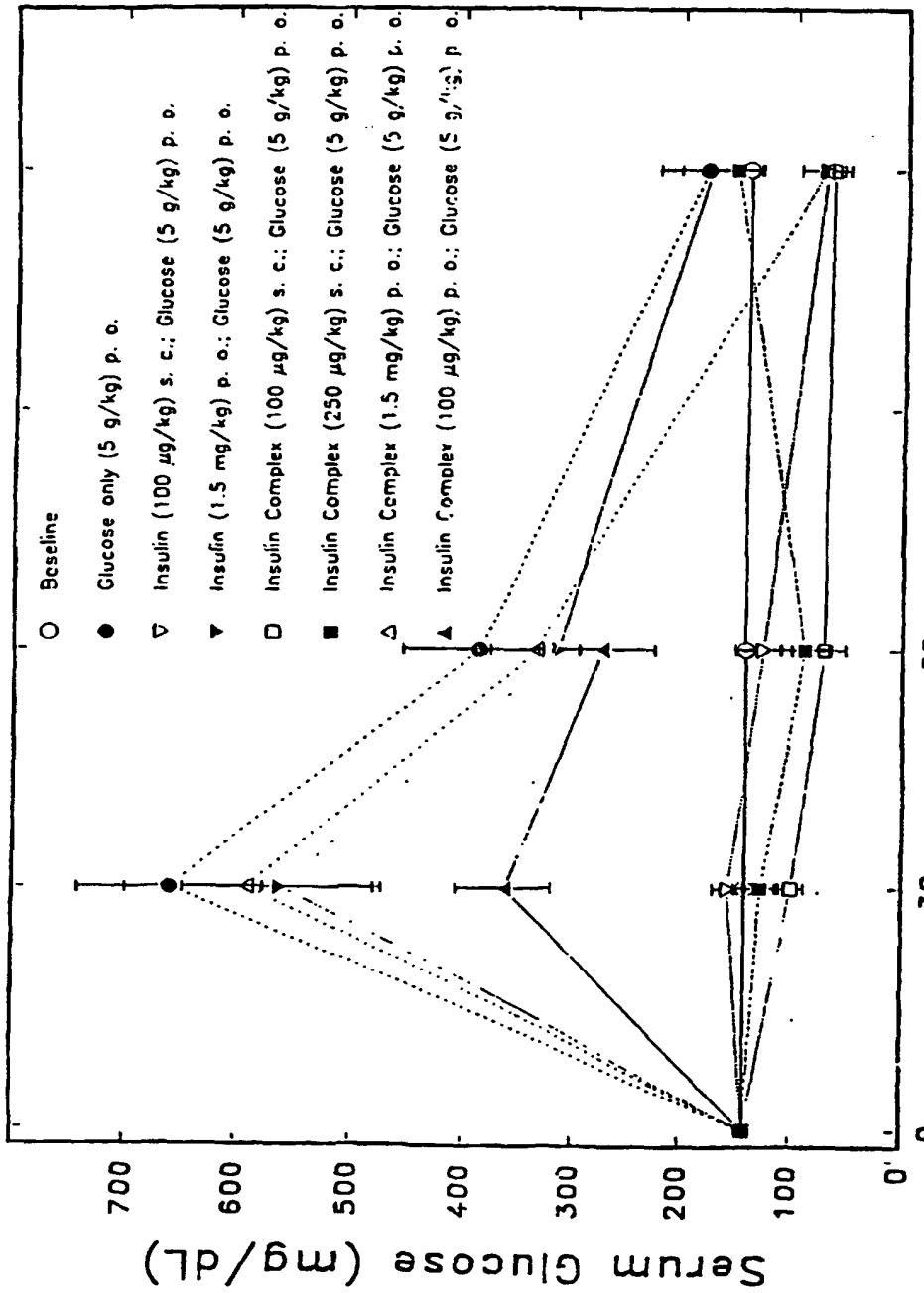

Serum Glucose (mg/dL)
700
600
500
400
300
200
100
0
0
30
60
90
120
Time (Minutes)
Baseline
Glucose only (5 g/kg) p. o.
Insulin (100 µg/kg) s. c.; Glucose (5 g/kg) p. o.
Insulin (1.5 mg/kg) p. o.; Glucose (5 g/kg) p. o.
Insulin Complex (100 µg/kg) s. c.; Glucose (5 g/kg) p. o.
Insulin Complex (250 µg/kg) s. c.; Glucose (5 g/kg) p. o.
Insulin Complex (1.5 mg/kg) p. o.; Glucose (5 g/kg) p. o.
Insulin Complex (100 µg/kg) p. o.; Glucose (5 g/kg) p. o.

FIG 2

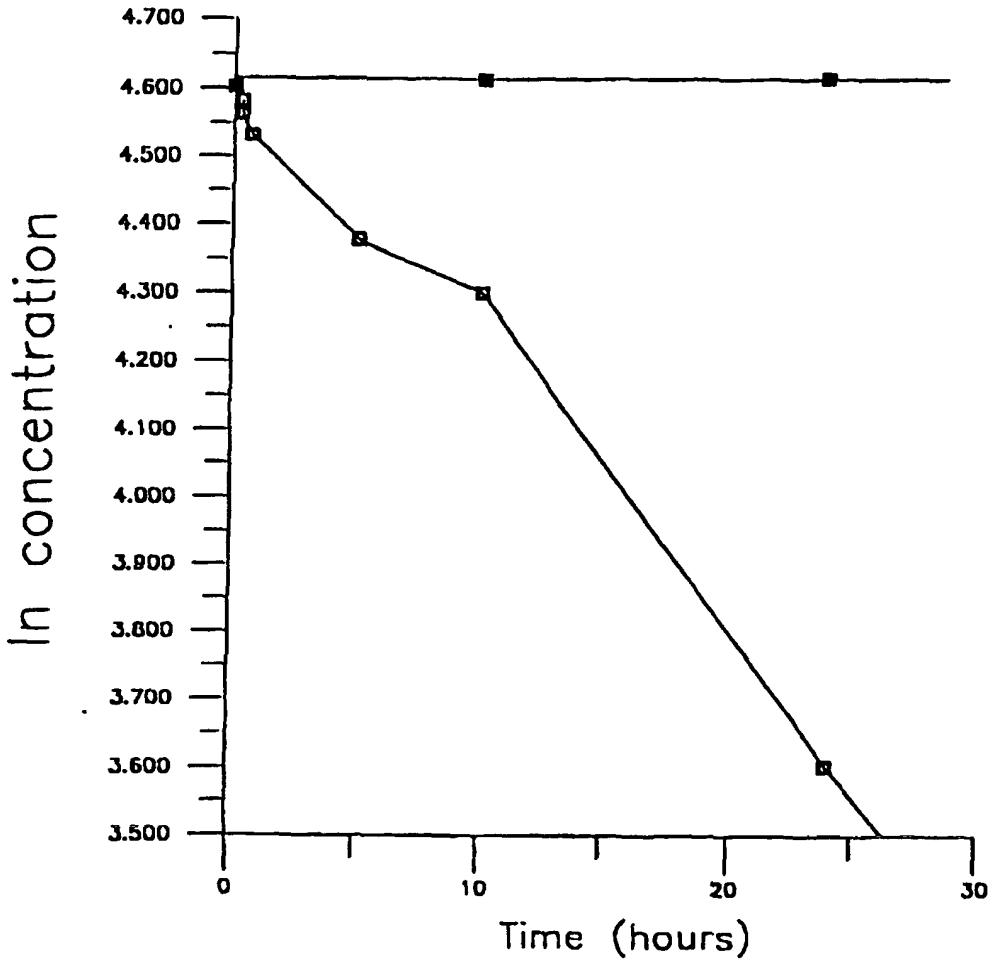

4.700
4.600
4.500
4.400
4.300
4.200
4.100
4.000
3.900
3.800
3.700
3.600
3.500
ln concentration
0
10
20
30
Time (hours)

Fig. 3

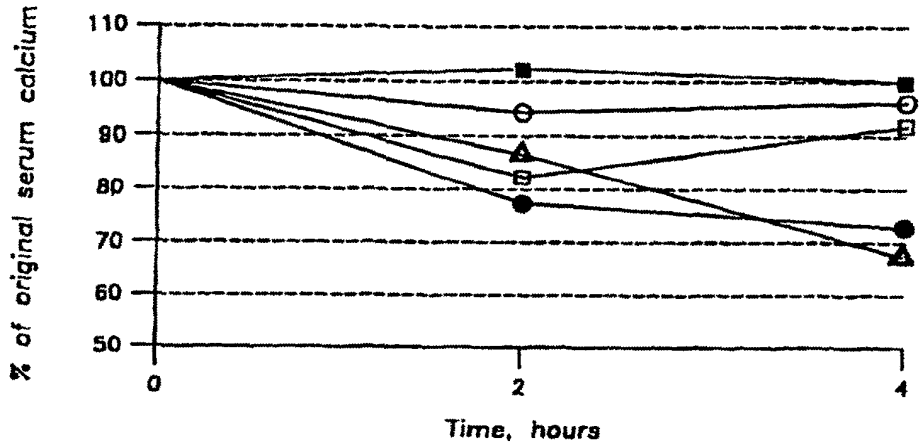

% of original serum calcium
110
100
90
80
70
60
50
0
2
4
Time, hours

Fig. 4

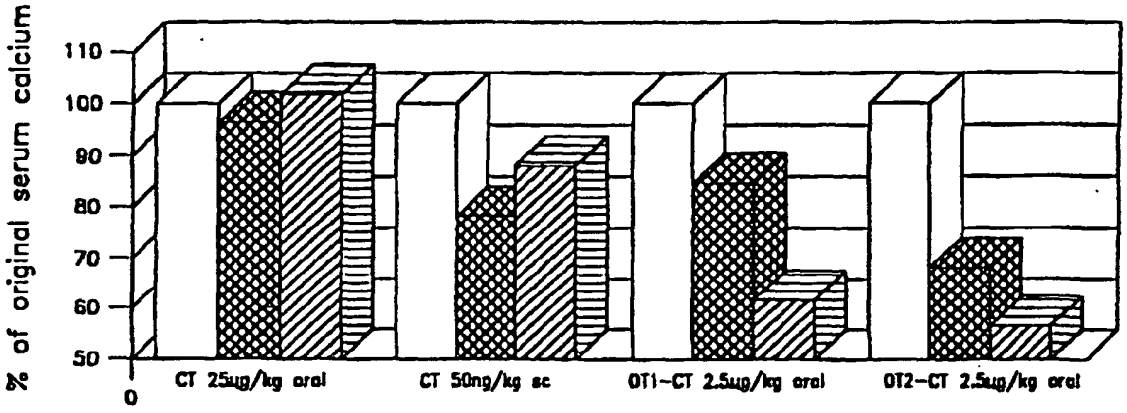

% of original serum calcium
110
100
90
80
70
60
50
0
CT 25ug/kg oral
CT 50ng/kg sc
OT1-CT 2.5ug/kg oral
OT2-CT 2.5ug/kg oral

Fig. 5

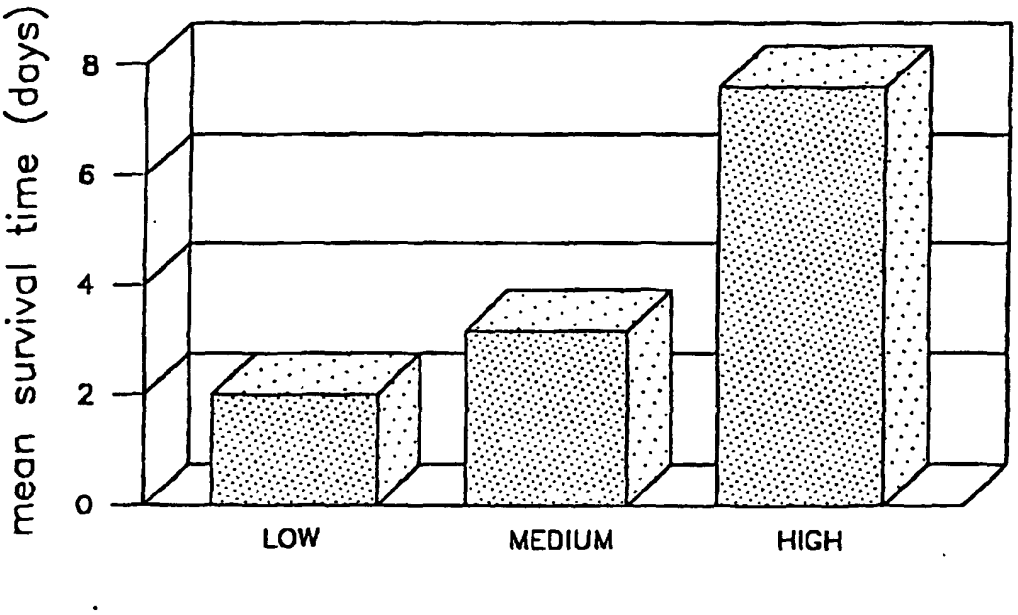


Fig. 6

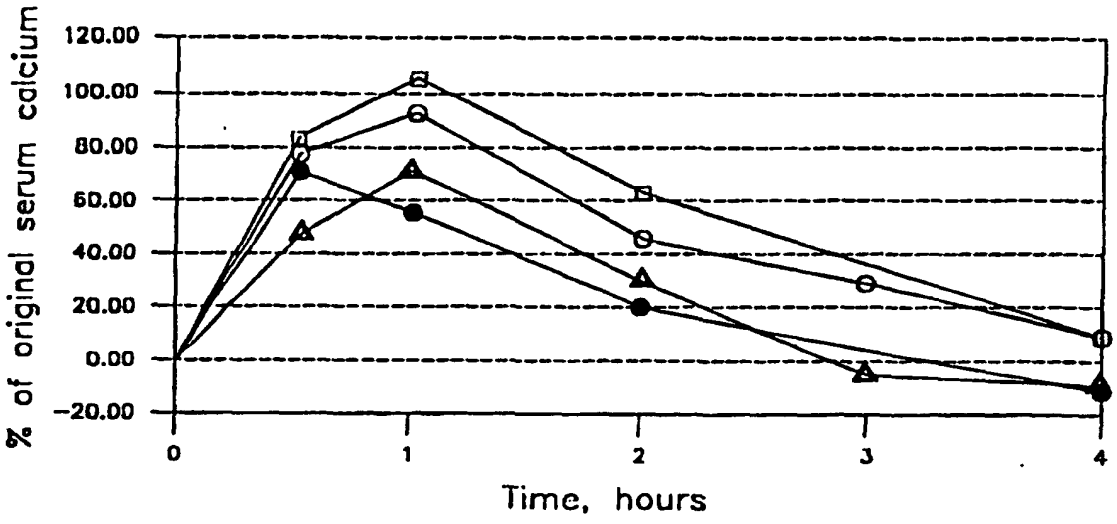

% of original serum calcium
120.00
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
0
1
2
3
4
Time, hours

Fig. 7

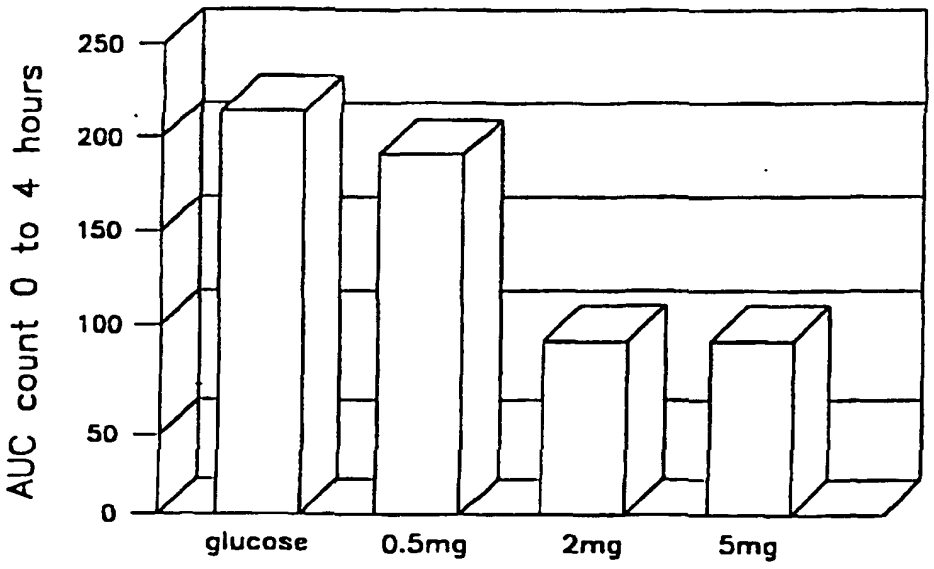


Fig. 8